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14. ABSTRACT

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. Cerebral edema, the abnormal accumulation of fluid within the brain parenchyma, contributes to elevated intracranial pressure (ICP) and is a common life-threatening neurological complication following TBI. Unfortunately, neurosurgical approaches to alleviate increased ICP remain controversial and medical therapies are lacking due in part to the absence of viable drug targets. In the present study, genetic inhibition (P2X7-/- mice) of the purinergic P2x7 receptor attenuated the expression of the pro-inflammatory cytokine, interleukin-1β (IL-1β) and reduced cerebral edema following controlled cortical impact, as compared to wild-type mice. Similarly, brilliant blue G (BBG), a clinically non-toxic P2X7 inhibitor, inhibited IL-1β expression, limited edemic development, and improved neurobehavioral outcomes after TBI. The beneficial effects of BBG followed either prophylactic administration via the drinking water for one week prior to injury or via an intravenous bolus administration up to four hours after TBI, suggesting a clinically-implementable therapeutic window. Notably, P2X7 localized within astrocytic end feet and administration of BBG decreased the expression of glial fibrillary acidic protein (GFAP), a reactive astrocyte marker, and attenuated the expression of aquaporin-4 (AQP4), an astrocytic water channel that promotes cellular edema. Together, these data implicate P2X7 as a novel therapeutic target to prevent secondary neurological injury after TBI, a finding that warrants further investigation.

15. SUBJECT TERMS
Traumatic brain injury (TBI), elevated intracranial pressure (ICP), secondary neurological injury, military deployment

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Abstract

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. Cerebral edema, the abnormal accumulation of fluid within the brain parenchyma, contributes to elevated intracranial pressure (ICP) and is a common life-threatening neurological complication following TBI. Unfortunately, neurosurgical approaches to alleviate increased ICP remain controversial and medical therapies are lacking due in part to the absence of viable drug targets. In the present study, genetic inhibition (P2X7-/- mice) of the purinergic P2x7 receptor attenuated the expression of the pro-inflammatory cytokine, interleukin-1β (IL-1β) and reduced cerebral edema following controlled cortical impact, as compared to wild-type mice. Similarly, brilliant blue G (BBG), a clinically non-toxic P2X7 inhibitor, inhibited IL-1β expression, limited edemic development, and improved neurobehavioral outcomes after TBI. The beneficial effects of BBG followed either prophylactic administration via the drinking water for one week prior to injury or via an intravenous bolus administration up to four hours after TBI, suggesting a clinically-implementable therapeutic window. Notably, P2X7 localized within astrocytic end feet and administration of BBG decreased the expression of glial fibrillary acidic protein (GFAP), a reactive astrocyte marker, and attenuated the expression of aquaporin-4 (AQP4), an astrocytic water channel that promotes cellular edema. Together, these data implicate P2X7 as a novel therapeutic target to prevent secondary neurological injury after TBI, a finding that warrants further investigation

TSNRP Research Priorities that Study or Project Addresses

Primary Priority

Force Health Protection:	☐ Fit and ready force ☐ Deploy with and care for the warrior ☐ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☒ Translating Knowledge & Research Findings into Practice in a Military Context

Progress towards Achievement of Specific Aims of the Study or Project

The overall objective of this research was to elucidate molecular and cellular mechanisms that promote cerebral edema, which may aid in the development of novel therapeutics to limit neurological dysfunction and reduce the incidence of neuropsychiatric sequelae following TBI. The purinergic receptor P2X7 has been implicated in the processing and or release of interleukin 1 beta (IL-1β), the prototypical, pro-inflammatory cytokine. A low affinity ATP activated receptor, P2X7 plays a role in the innate immunity and upon activation causes trans-membrane ion fluxes and formation of membrane pores as well as its role in the production and release of IL-1β. Others have shown a positive correlative role between IL-1β and the development of cerebral edema after brain injury. To this end we proposed a mechanistic hypothesis to explain the etiology of cerebral edema, which if shown, could potentially lead to new therapies in TBI.

Specific Aim 1: To establish the cellular localization and temporal pattern of P2X7 expression following TBI.

Hypothesis. *The purinergic receptor, P2X7, is activated following TBI.*

Rationale. The P2X7 receptor is reportedly distributed on cells of the immune system including macrophages, monocytes, lymphocytes, etc; additionally, the receptor is found in glia cells of the central and peripheral nervous system as well as spinal cord neurons (Coddou, Yan, Obsil, Huidobro-Toro, & Stojilkovic, 2011; Wang, et al., 2004). Cellular localization of the P2X7 receptor has yet to be clearly defined after TBI; therefore these studies will show, for the first time, the temporal pattern and cellular localization of P2X7 in the brain following TBI.

The expression of P2X7, the presumed cellular target of BBG action, was next assessed within the brain. P2X7 was basally expressed within the cerebral cortex, as demonstrated by Western blotting; however, expression was not increased following TBI, as compared to sham-operated mice (**Figure 1**).

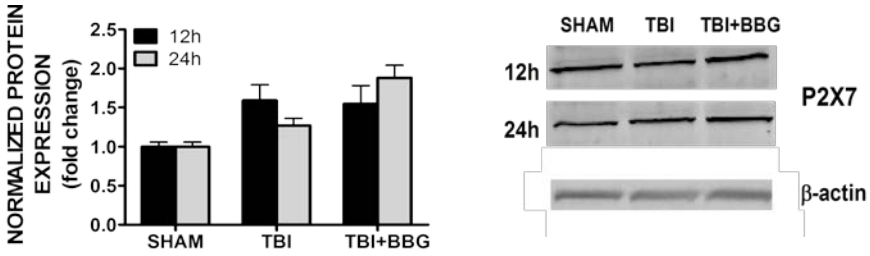
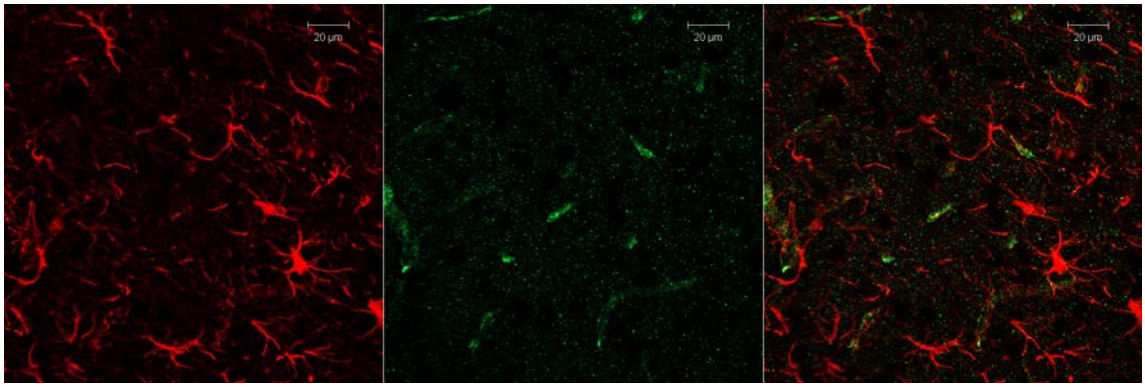


Figure 1: Western blot analysis of P2X7 receptor after TBI. Representative Western blots (top panel) of P2X7 in the cerebral cortex of mice following sham injury, TBI, or TBI + 50 mg/kg BBG. Tissue was collected at 12h or 24h after TBI. Blots were normalized to β-actin to control for equal protein loading between lanes. Data are representative of six mice/group. Densitometric analysis of Western blots (bottom panel) is presented as P2x7 expression following normalization to β-actin

Immunohistochemical analysis revealed that P2X7 strongly co-localized with the astrocytic endfoot marker, aquaporin-4 (AQP4) (**Figure 2b**) whereas dual labeling was not observed with the microglial marker, CD11b (**Figure 2c**) or the astrocytic cell body marker, glial fibrillary acidic protein (GFAP) (**Figure 2a**). Furthermore, no localization was observed between P2X7 and the neuron-specific marker, NeuN (data not shown). Together, these data implicate astrocytes as a key mediator of the biological actions of P2X7 and as a possible cellular target of BBG after TBI.

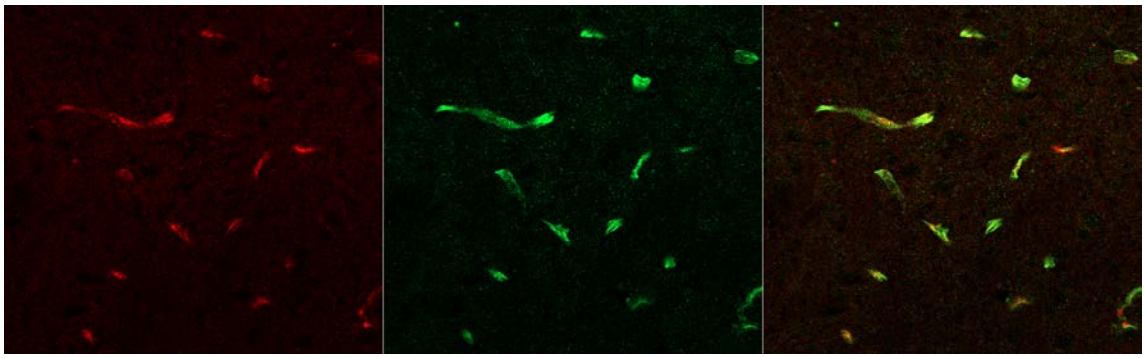
A.

GFAP P2X7 Merge



B.

AQP4 P2X7 Merge



C.

P2X7 CD11B Merge

Specific Aim 2: To establish whether inhibition of P2X7 decreases cerebral edema following TBI.

Hypothesis. *Antagonism of the purinergic receptor, P2X7, will reduce cerebral edema after TBI.*

Rationale. ATP is generally contained to intracellular compartments and is not commonly found in the external milieu. After injury, ATP is released into the external environment where it can act upon the low affinity purinergic receptor P2X7. The P2X7 receptor has been implicated in neuropathic pain and inflammatory responses throughout the body. Upon P2X7 well known to open channels that are that are permeable to both mono and divalent cations, altering concentrations that can fluxes in water content. Work by Nedergaard and colleagues showed that doses of 10mg/kg and 50mg/kg of BBG, a specific P2X7 antagonist, showed improvements after spinal cord injury. The proper dose and effect of BBG in the brain remains largely unexplored; therefore, these studies will implicate P2x7 in edema development after TBI. If successful, these studies will also identify a novel therapeutic use for brilliant blue G (BBG), a clinically- safe P2X7 antagonist, amendable to implementation on the battlefield (Wang, et al., 2004).

BBG reduces post-traumatic cerebral edema with an extended therapeutic window

Brain water content, a sensitive measure of cerebral edema, was significantly increased within the ipsilateral cortex at 24h post-TBI (83.6 ± 0.4% brain water content after TBI vs. 77.9 ± 0.2% in sham, p<0.001 vs. sham) (**Figure 3**). A single, intravenous injection of 50 mg/kg BBG at 15 minutes prior to injury attenuated brain water content after TBI (80.6 ± 0.5%; p<0.01 vs. TBI) whereas administration of 25 mg/kg BBG did not significantly reduce edema (83.3% ± 0.3%; not significantly different from TBI). Notably, the ability of 100 mg/kg BBG to reduce edema was not significantly different from administration of 50 mg/kg (81.0 ± 0.2%; p<0.001 vs. TBI), suggesting 50 mg/kg was the lowest efficacious dose to limit edemic development after TBI (**Figure 4**). For all studies, brain water content within the contralateral (uninjured) cortices did not significantly differ between any of the treatment groups (data not shown). Furthermore, administration of BBG alone (50 mg/kg, i.v., 15 minute pre-treatment) did not significantly change brain water content, as compared to placebo-treated, sham-operated mice (**Figure 3**), suggesting an injury-specific reduction in edema.

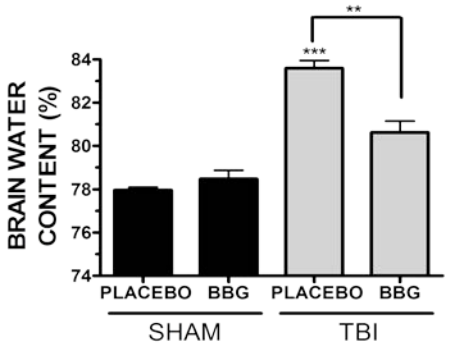


Figure 3: Antagonism of P2X7 reduces cerebral edema after TBI.
A single intravenous bolus of 50 mg/kg BBG provided 15 minutes prior to TBI, significantly reduced the development of cerebral edema at 24h post-TBI, as measured by brain water content. Comparisons within each hemisphere between different treatments groups were done using a one-way ANOVA followed by Dunnett's post-hoc test (* p<0.05, ** p<0.01, ***p<0.001 vs. the ipsilateral hemisphere in sham-operated mice). No significant differences in cerebral edema were observed between groups in the contralateral hemisphere. Data are represented as the mean ± SEM from 5-6 mice/group

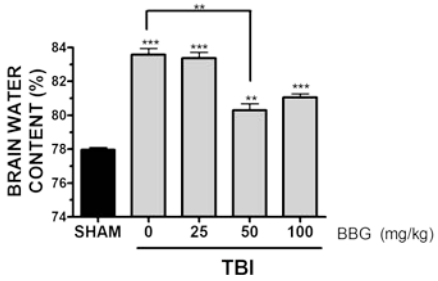


Figure 4: Dose determination of BBG for antagonism of P2X7 after TBI. A single intravenous bolus of 50-100 mg/kg BBG administered 0.5h after TBI significantly reduced cerebral edema at 24h post-TBI. . Comparisons within each hemisphere between different treatments groups were done using a one-way ANOVA followed by Dunnett's post-hoc test (* p<0.05, ** p<0.01, ***p<0.001 vs. the ipsilateral hemisphere in sham-operated mice). No significant differences in cerebral edema were observed between groups in the contralateral hemisphere. Data are represented as the mean ± SEM from 5-6 mice/group

The therapeutic window whereby BBG reduced edemic development was next established. A 1h post-treatment with 50 mg/kg significantly reduce cerebral edema (81.3 ± 0.2%, $p<0.05$ vs. TBI) to a similar extent as pre-treatment (**Figure 5**; see **Figure 3** for comparison). Similarly, a 4h post-treatment effectively attenuated post-traumatic edema (81.4 ± 0.4%, $p<0.05$ vs. TBI, no significantly different from 1h post-treatment). In contrast, 8h post-treatment with 50 mg/kg was ineffective at reducing edema, as compared to TBI (83.2% ± 0.2%), suggesting a 4h post-injury therapeutic window.

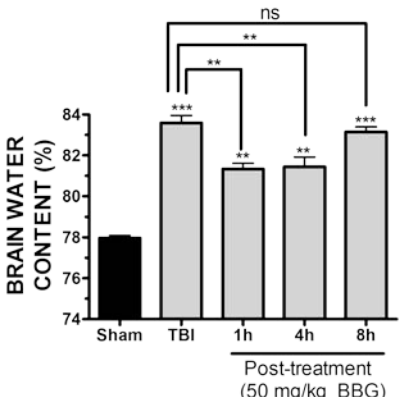


Figure 5: Determination of BBG time of treatment after TBI.
Administration of a single intravenous bolus of 50 mg/kg BBG reduced cerebral edema when administered 1h or 4h after injury. This effect was lost if post-treatment was delayed beyond 8h from the time of injury. Comparisons within each hemisphere between different treatments groups were done using a one-way ANOVA followed by Dunnett's post-hoc test (* $p<0.05$, ** $p<0.01$, ***$p<0.001$ vs. the ipsilateral hemisphere in sham-operated mice). No significant differences in cerebral edema were observed between groups in the contralateral hemisphere. Data are represented as the mean ± SEM from 5-6 mice/group

We next determined whether prophylactic administration of BBG may reduce edema. Oral administration of 25 mg/mL BBG via the drinking water for one week prior to injury effectively decreased brain edema after TBI (80.9 ± 0.2%, $p<0.01$ vs. TBI) (**Figure 6**). In contrast, 10 mg/mL BBG via the drinking water did not significantly reduce edema, as compared to mice receiving water containing only placebo. As a whole, either prophylactic oral administration or post-injury intravenous administration of BBG effective attenuates brain edema after TBI.

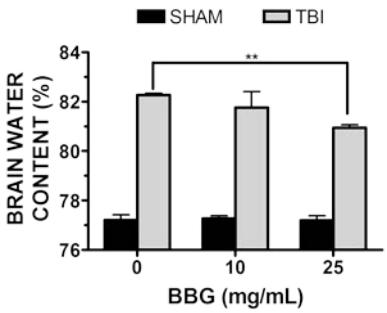


Figure 6: Prophylactic treatment with BBG reduces cerebral edema after TBI. Prophylactic treatment with BBG in the drinking water for 7 days reduced edema at 24h post-TBI at a concentration of 25 mg/ml but not 10mg/ml. Comparisons within each hemisphere between different treatments groups were done using a one-way ANOVA followed by Dunnett's post-hoc test (* $p<0.05$, ** $p<0.01$, ***$p<0.001$ vs. the ipsilateral hemisphere in sham-operated mice). No significant differences in cerebral edema were observed between groups in the contralateral hemisphere. Data are represented as the mean ± SEM from 5-6 mice/group

Brain expression of P2X7 after TBI

Peripheral administration of BBG reduced brain edema, although the potential tissue and cellular targets of BBG remained unclear. Intravenous administration of 50 mg/kg BBG produced a transient deep blue color over the first 24h within the eyes, nose, ears, and paws (**Figure 7a**), demonstrating wide peripheral distribution throughout the circulatory system. No trace of blue color was observed by 72h post-administration. Similarly, oral administration of BBG for one week via the drinking water also produced a faint blue hue in the paws and eyes, albeit to a far lesser extent, as compared to intravenous administration. Consistent with the observed blue appearance, serum levels of BBG reached 383 ± 33.3 μM and 1.73 ± 0.07 mM following intravenous administration of 50 mg/kg and 100 mg/kg, respectively. Whether BBG acted peripherally or crossed the blood-brain barrier to directly affect the brain after TBI remained unclear. Consistent with a potential direct effect, the brains of mice administered BBG appeared greyish-blue, with blue color observed within the cerebral vasculature and brain tissue. Most notably, the contused cortex exhibited a distinct blue color (**Figure 7b**), suggesting BBG can enter the brain and preferentially accumulates at high levels around damaged tissue, presumably where the blood-brain barrier is disrupted.

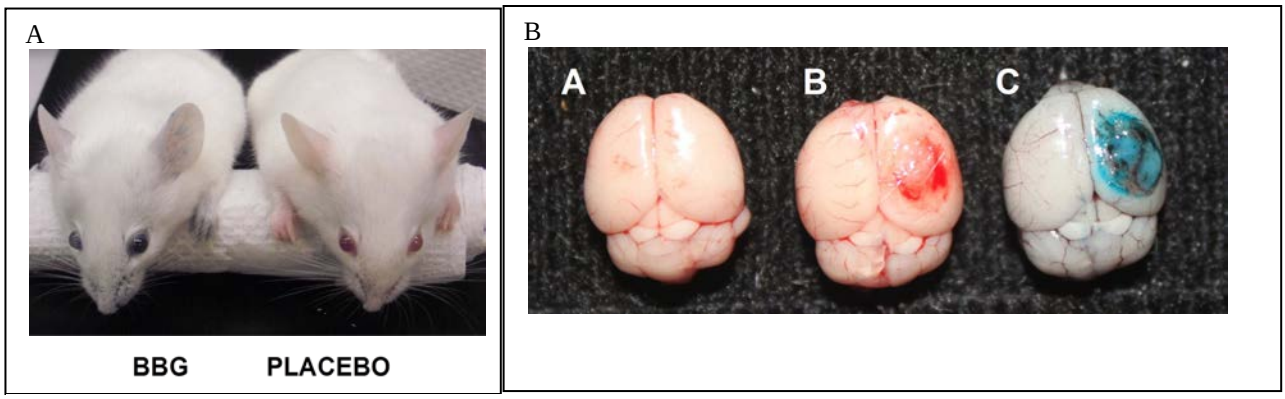


Figure 7: Distribution of BBG after TBI. **(A)** Photograph of representative mice following an intravenous administration of placebo (right) and a BBG (50 mg/kg; left). Note the blue appearance in the skin, eyes, ears, paws and tail. **(B)** BBG accumulates in the contused cortex after TBI. Photographs of brains taken from a sham-operated mouse administered placebo (left panel), a mouse administered placebo at 0.5h after TBI (middle panel), or a mouse administered 50 mg/kg BBG via the tail vein at 0.5h post-TBI.

P2X7-/- mice exhibit reduced cerebral edema after TBI

BBG is a highly selective inhibitor of P2X7; however, pharmacological agents often exhibit "off-target" or non-specific effects. To validate P2X7 as a potential therapeutic target to reduce brain edema, we next performed studies in P2X7-/- mice. Consistent with data collected after BBG administration, P2X7-/- mice exhibited a significant reduction in brain water content, as compared to wild-type mice, following TBI (81.0 ± 0.4% in P2X7-/- vs. 83.7 ± 0.3% in wild-type; $p<0.01$) (**Figure 8**). These findings were supported by the measurement of edemic volume in living mice using MRI. P2X7-/- mice exhibited a 36% reduced in edemic volume after TBI, as compared to wild-type mice (14.4 ± 0.7 mm^3 in wild-type mice vs. 9.2 ± 1.5 mm^3 in P2X7-/- mice; $p<0.01$ vs. wild-type) (**Figure 9**). Brain water content was not significantly different either in sham-operated mice (**Figure 9**) or in the contralateral hemisphere of wild-type or P2X7-/- mice.

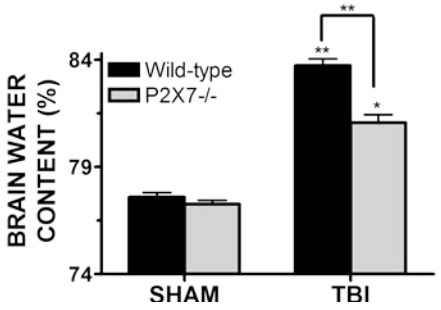
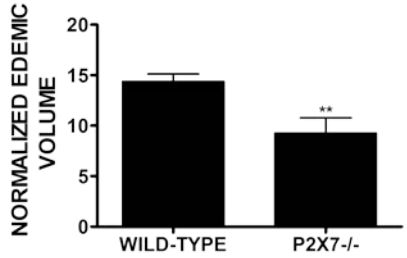


Figure 8: Brain water measurement of genetic inhibition of P2X7 after TBI. P2X7-/- mice exhibited a significant reduction in brain water content, as compared to wild-type mice, when assessed at 24h post-TBI. Comparisons within each hemisphere between different treatments groups were done using a one-way ANOVA followed by Dunnett's post-hoc test (* $p<0.05$ vs. the ipsilateral hemisphere in sham-operated mice). No significant differences in cerebral edema were observed between groups in the contralateral hemisphere.



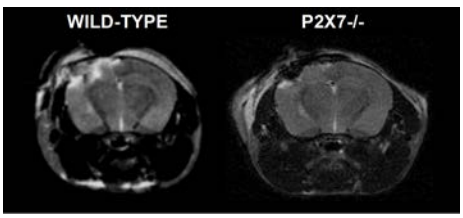
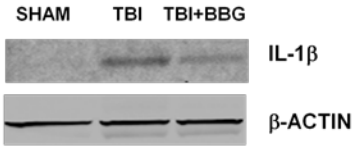


Figure 9: Genetic inhibition of P2X7 attenuates cerebral edema after TBI as measured by MRI. P2X7-/- mice displayed attenuated cerebral edema, as compared to wild-type mice, when assessed by MRI. The top panels depict a representative wild-type and a P2X7-/- mouse imaged at 24h post-TBI. Bottom panels represent the mean edemic volume of mice imaged by MRI. Data are represented as the mean ± SEM from six mice/group and were analyzed using a t-test ($p<0.01$ vs. wild-type).

Specific Aim 3: To determine whether P2x7 inhibition reduces IL1-1β production and release following TBI.

Hypothesis. Inhibition of the P2X7 receptor will decrease the production of IL-1β following TBI

Rationale. The brain has long been thought to be immuno-privileged. Recent work by numerous laboratories has shown that the innate immune system functions similarly if not identically in the brain as in the periphery. The purinergic receptor P2X7, originally described in cells of hematopoetic origin to include microglia, is known to not only allow the bidirectional flow of cations but to have an important role in the release of proinflammatory cytokines such as IL-1β. IL-1β is an important mediator in chronic pain, inflammation and neurodegeneration and can affect neuronal cell death after injuries such as TBI. P2X7 activity has been reported to have a role in the pathology of disease processes such as depression by regulating the release of the proinflammatory cytokine IL-1β. Ito et al (1996) describes a positive correlation between Il-1β in the CSF, cerebral edema and negative outcomes associated with TBI. This provides the theoretical link between the immune system and neurologic injuries providing the foundation for the studies of this aim. Therefore, these studies will implicate P2X7 activation in the production and release of the pro- inflammatory cytokine IL-1β after TBI, suggesting an innate immune component of the inflammatory pathway of TBI and cerebral edema (Skaper, et al., 2010; Wang, et al., 2004).



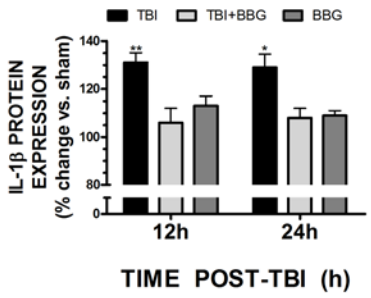


Figure 10: Western blot analysis of IL-1β after inhibition of P2X7 . A single intravenous bolus of 50-100 mg/kg BBG administered 0.5h after TBI significantly reduced peri-contusional IL-1β expression, as assessed by **(B)** EIA and by **(A)** Western blotting at 12h or 24h post-injury. In panel A,data are represented as IL-1β expression as a % of sham expression levels. In panel B, data was normalized to β-actin to control for equal protein loading between lanes. Data are representative of six mice/group. Data were analyzed with One-Way ANOVA followed by Dunnett's post-hoc test (* $p<0.05$, ** $p<0.01$ vs. sham operated mice).

P2X7 mediates glial reactivity after TBI

IL-1β induces reactive astrogliosis after TBI; therefore, the ability of BBG to attenuate the expression of GFAP, a hallmark of gliosis, was next assessed. GFAP expression was significantly increased by 299.7 ± 72.2% within the peri-contusional cortex ($p<0.05$ vs. sham) and 222.0 ± 28.6% ($p<0.01$ vs. sham) of sham-operated mice at 12h and 24h post-TBI, respectively (**Figure 11**). Post-treatment with 50 mg/kg BBG reduced GFAP expression to 216.1 ± 88.6% (not significantly different from either sham or TBI) and 145.3 ± 15.2% ($p<0.05$ vs. TBI, not significantly different from sham) of expression levels in sham-operated mice at 12h and 24h, respectively.

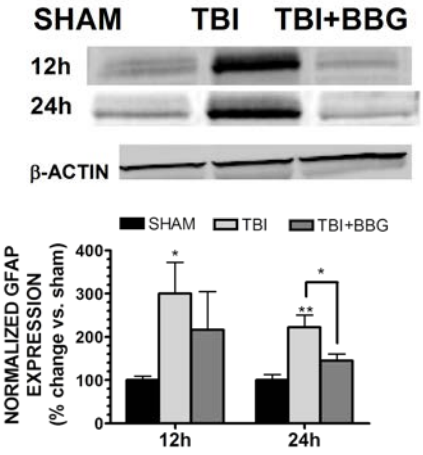


Figure 11: BBG attenuates glial activation as measured by GFAP expression. Representative Western blot (top panel) of cortical GFAP expression taken at 12h or24h after sham injury, TBI, or TBI + 50 mg/kg BBG. Data (mean ± SEM) are representative of six mice/group from three independent experiments (n=3/group in each experiment) and are expressed as % change vs. sham. Data were analyzed by One-Way ANOVA followed by Dunnett's post-hoc test (* $p<0.05$, ** $p<0.01$ vs. sham operated mice).

Consistent with the inhibitory effect of BBG on post-traumatic cerebral edema and glial reactivity, BBG attenuated the expression of the astrocytic water channel, AQP4, after TBI. AQP4 protein expression was increased within the pericontusional context at 12h (1.7 ± 0.1 fold increase; $p<0.01$ vs. sham) and at 24h (1.5 ± 0.1 fold increase; $p<0.05$ vs. sham) after TBI. Intravenous administration of 50 mg/kg BBG at 0.5h post-injury attenuated the

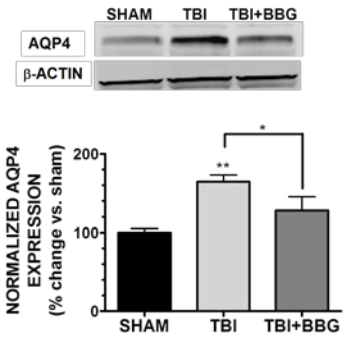


Figure 12: Western blot analysis of AQP4 expression after P2X7 antagonism. Representative Western blot (top panel) of AQP4 in the cerebral cortex of mice at 12h following sham injury, TBI, or TBI + 50 mg/kg BBG. Densitometric analysis of Western blots (bottom panels) is presented as AQP4 expression following normalization to β-actin, which was used to control for equal protein loading. Data (mean ± SEM) are representative of six mice/group from three independent experiments (n=3/group in each experiment) and are expressed as % change vs. sham. Data were analyzed by One-Way ANOVA followed by Dunnett's post-hoc test (* $p<0.05$, ** $p<0.01$ vs. sham operated mice).

TBI-induced increases in AQP4 expression (1.3 ± 0.2 and 1.1 ± 0.1 fold increase vs. sham at 12h and 24h, respectively; $p<0.05$ vs. TBI, not significant different from sham) (**Figure 12**).

Specific Aim 4: To determine whether inhibition of P2X7 improves neurologic outcomes following TBI

Hypothesis. *Inhibition of the P2X7 receptor after TBI will improve neurologic outcomes*

Rationale. The brain has long been thought to be immuno-privileged. Recent work by numerous laboratories has shown that the innate immune system functions similarly if not identically in the brain as in the periphery. The purinergic receptor P2X7, originally described in cells of hematopoetic origin to include microglia, is known to not only allow the bidirectional flow of cations but to have an important role in the release of proinflammatory cytokines such as IL-1β. IL-1β is an important mediator in chronic pain, inflammation and neurodegeneration and can affect neuronal cell death after injuries such as TBI. P2X7 activity has been reported to have a role in the pathology of disease processes such as depression by regulating the release of the proinflammatory cytokine IL-1β. P2X7 inhibition has been reported to improve outcomes after both spinal cord injury and stroke(Arbeloa, Perez-Samartin, Gottlieb, & Matute, 2011; Skaper, et al., 2010; Wang, et al., 2004). Therefore, we propose that these studies will show for the first time that inhibition of P2X7 will improve neurobehavioral outcomes after TBI.

BBG improves neurobehavioral outcomes after TBI

Depression and anxiety disorders are common psychiatric co-morbidities after a TBI. Thus, the ability of BBG to reduce neuropsychiatric dysfunction was next explored. A significant increase in open-field hyperlocomotion (total number of squares entered) was observed following TBI ($p<0.01$ vs. sham) (**Figure 13**). Administration of 50 mg/kg BBG

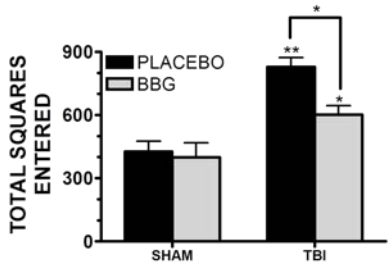


Figure 13: BBG improves open field hyperlocomotion after TBI. Post-injury administration of 50 mg/kg BBG significantly attenuated post-traumatic hyperlocomotion following TBI in the open field test and (B) time to first immobility in the forced swim test, a sensitive estimate of depressive like behavior, as compared to placebo-treated mice. Data are expressed as the mean ± SEM from 10-12 mice/group and were compared by One-Way ANOVA followed by Dunnett's post-hoc test (* $p<0.05$, ** $p<0.01$ vs. sham operated mice).

partially resolved the increase in post-traumatic hyperlocomotion by ~50% ($p<0.05$ vs. sham and TBI). In contrast, BBG administration had no significant effect on basal activity in sham-operated mice.

Following TBI, mice exhibited a reduced time to latency to develop behavioral despair, a measure of depression, using the forced swim test. Sham-operated mice displayed a latency of 70.8 ± 8.3s whereas TBI reduced this time to 44.5 ± 7.4s ($p<0.05$ vs. sham). Post-injury administration of 50 mg/kg BBG significantly increased the latency time to 85.4 ± 5.5s ($p<0.01$ vs. TBI, not significantly different from sham). (**Figure 14**). Notably, BBG administration did not significantly change the latency time in sham-operated mice, suggesting an injury specific effect.

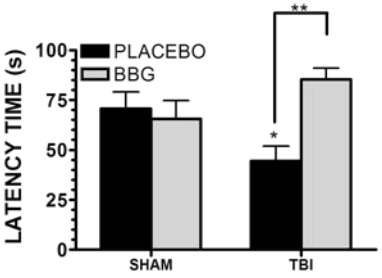


Figure 14: BBG improves depressive effects after TBI. Post-injury administration of 50 mg/kg BBG significantly increased time to first immobility in the forced swim test, a sensitive estimate of depressive like behavior, as compared to placebo-treated mice. Data are expressed as the mean ± SEM from 10-12 mice/group and were compared by One-Way ANOVA followed by Dunnett's post-hoc test (* $p<0.05$, ** $p<0.01$ vs. sham operated mice).

DISCUSSION

Preventative measures reduce the incidence and/or severity of TBI, yet one-third of hospitalized TBI patients die from injuries that are secondary to the initial trauma. The development of post-traumatic edema promotes clinical deterioration and worsens long-term outcomes, at least in part, by limiting cerebral perfusion, by increasing brain herniation, and by increasing the manifestation of neuropsychiatric impairments such as headaches, anxiety, depression, sleep disturbances, and appetite loss (H. S. Levin, et al., 1991; Rogers & Read, 2007; Saul & Ducker, 1982a; Whelan-Goodinson, Ponsford,

Johnston, & Grant, 2009). Thus, elucidation of the cellular mechanisms of neurological injury may permit the development of efficacious therapeutics to improve patient outcomes after TBI.

In the present study, genetic (P2X7-/-) or pharmacological (BBG) inhibition of P2X7 reduced secondary brain injury and improved functional outcomes after a moderate TBI in mice. BBG, a FDA-approved, water soluble, structural and functional analogue of FD&C blue dye No. 1 (also called Brilliant blue FCF or E133), is a widely used food additive and coloring agent that exhibits no toxicity at doses up to 1g/kg/d in humans (Register, 2006). Herein, BBG reduced peri-contusional IL-1β, limited AQP4 expression, attenuated edemic development, and improved neurobehavioral outcomes. These beneficial effects were observed whether BBG was intravenously administered as a single bolus up to four hours after injury or chronically administered via the drinking water. Thus, clinically safe doses of BBG may reduce neurological injury after TBI, either via a clinically-implementable post-injury temporal window or via prophylactic administration.

Cellular edema is the predominant form of edema during the acute and sub-acute phase after TBI (Bullock, Maxwell, Graham, Teasdale, & Adams, 1991; J. Ito, et al., 1996). Astrocytic swelling, a characteristic feature of cellular edema, commenced within the first hours after head trauma in humans (Bullock, et al., 1991; J. Ito, et al., 1996) and glial activation temporally paralleled edemic development in pre-clinical models of TBI (Dietrich, et al., 1999; Hinkle, et al., 1997). Furthermore, increased serum and CSF levels of the activated astrocyte markers, S100β and GFAP, directly correlated with patient outcomes after TBI (Hayakata, et al., 2004; Pelinka, Kroepfl, Leixnering, et al., 2004; Pelinka, Kroepfl, Schmidhammer, et al., 2004), supporting a possible role for astrocytes in the genesis of secondary neurovascular injury; however, controversy remains as to whether

astrocytes exert beneficial and/or detrimental functions after brain injury (Laird, Vender, & Dhandapani, 2008). Along these lines, astrocytes are the predominant cell type within the neurovascular unit, providing trophic support for neurons, regulating cerebral blood flow, and maintaining ionic and neurotransmitter homeostasis under physiological conditions. Conversely, astrocytes may generate cerebral innate immune responses after injury or infection, releasing pro-inflammatory mediators (Farina, et al., 2007).

AQP4, a bidirectional water channel expressed in the perivascular end feet of astrocytes, mediated glial swelling *in vitro* and was associated with the development of cellular edema after TBI in humans and rodents (Badaut, et al., 2011; Hu, et al., 2005). Although causative studies remain unperformed after neurotrauma, attenuated swelling of pericapillary astrocytic foot processes, decreased cellular edema, and reduced mortality were observed in AQP4-deficient mice after ischemic stroke or acute water intoxication (Manley, et al., 2000). Additionally, genetic deletion of AQP4 attenuated astrocytic migration and glial scar formation, implicating AQP4 as a potential therapeutic target to restrict deleterious astrocytic responses to injury (Saadoun, et al., 2005). Unfortunately, clinically-efficacious drugs to inhibit AQP4 expression/function do not currently exist, at least in part, due to the limited understanding of AQP4 regulation at the cellular level. Notably, we and others recently identified IL-1β as a positive regulator of AQP4 expression in cultured astrocytes and in the mouse cerebral cortex (H. Ito, et al., 2006; Laird, et al., 2010). IL-1β expression is rapidly increased following brain insults and functionally promotes reactive astrogliosis after penetrating brain injury (Lin, et al., 2006). Furthermore, elevated concentrations of IL-1β in the CSF of head trauma patients correlated with an unfavorable clinical outcome (Chiaretti, et al., 2005; Hayakata, et al., 2004). Based on these findings, we hypothesized

that strategies which reduce post-traumatic IL-1β may effectively limit neurovascular injury after TBI.

IL-1β is synthesized as a biologically inactive 31-kDa precursor protein that requires proteolytic cleavage to generate the mature, biologically-active 17.5 kDa protein (Perregaux & Gabel, 1998). Expression of caspase-1 (also called interleukin-1 converting enzyme; ICE), the principal enzyme involved in the processing of pro-IL-1β into the mature IL-1β form, was upregulated within the rat forebrain after fluid percussion injury (Yakovlev, et al., 1997). Activated caspase-1 was strongly increased in brain tissue resected from both pediatric and adult TBI patients whereas pro-caspase-1 exhibited a decrease in expression as compared to control patients (Clark, et al., 1999; Satchell, et al., 2005). Furthermore, activated caspase-1 was elevated within the CSF of pediatric TBI patients, an observation that directly correlated with a concomitant increase in IL-1β and reduction in pro-IL-1β in these same patients (Satchell, et al., 2005). Functionally, genetic or pharmacological inhibition of caspase-1 reduced secondary tissue damage after experimental TBI in mice (Fink, et al., 1999). Taken together, these findings suggest clinical significance for caspase-1 activation after TBI and imply therapeutic targeting of caspase-1 pathway may improve outcomes.

The precise cellular mechanisms underlying caspase-1 activation remain poorly defined; however, repetitive or prolonged exposure to high concentrations of ATP increased the activation and the externalization of caspase-1 and promoted the formation of a large membrane pore required for the extracellular release of IL-1β (Laliberte, Eggler, & Gabel, 1999; Mariathasan, et al., 2006). ATP, an intracellular energy source under physiological conditions, is rapidly released into the extracellular space after traumatic or ischemic

injuries (Khakh & North, 2006; Latini, Corsi, Pedata, & Pepeu, 1996; Peng, et al., 2009; Ralevic & Burnstock, 1998). Although the functional significance remains poorly defined, the release of extracellular ATP promoted secondary tissue damage after traumatic spinal cord injury (Peng, et al., 2009). Furthermore, elevated levels of ATP metabolites within the CSF of a head trauma patient correlated with edemic development and elevated ICP (Cristofori, et al., 2005), implying a detrimental role for purinergic signaling after neurological injury.

The biological actions of ATP are mediated, at least in part, by activation of either metabotropic P2Y receptors or ionotropic P2X receptors (Ralevic & Burnstock, 1998). Among the purine receptor family members, P2X7 is a low-affinity receptor that preferentially responds to sustained elevations in ATP such as those which occurs after trauma, suggesting P2X7 possesses the optimal biophysical properties for mediating the detrimental actions of ATP after a brain injury. Herein, P2X7 specifically co-localized within astrocytic end feet within the brain, directly overlapping with the expression of AQP4. Consistent with a report showing extracellular ATP induced stellation and increased GFAP expression in astrocyte cultures (Neary, Baker, Jorgensen, & Norenberg, 1994), clinically-achievable doses of BBG decreased IL-1β production, reduced astrocytic activation, as assessed by GFAP expression, attenuated AQP4 expression, and limited cerebral edema after TBI in mice. Given the importance of cerebral edema and elevated ICP in patient mortality and long-term morbidity after TBI, P2X7 antagonism may improve acute clinical outcomes following TBI.

Increased rates of depression, aggression, and anxiety are observed over the first year in up to 51% of TBI survivors (Fann, et al., 2004); yet, a recent meta-analysis of 223 pre-clinical trials failed to identify any single intervention that significantly improved these

neurological outcomes after TBI (Wheaton, Mathias, & Vink, 2011). Interestingly, patients with idiopathic intracranial hypertension, a neurological disorder characterized by non-traumatic elevations in ICP, exhibited higher rates of developing depression and anxiety, as compared to matched control patients (Kleinschmidt, Digre, & Hanover, 2000). These clinical findings suggested post-traumatic elevations in ICP could directly induce psychiatric co-morbidities. IL-1β, which clinically correlates with elevated ICP after TBI (Chiaretti, et al., 2005; Holmin & Hojeberg, 2004; Shiozaki, et al., 2005), is implicated in the pathophysiology of depressive and anxiety (Koo & Duman, 2008, 2009a, 2009b; Norman, et al., 2010). Thus, the production of IL-1β may provide a key mechanistic bridge between acute traumatic injury and long-term neurological outcomes. Consistent with this notion, post-injury administration of clinically-relevant doses of BBG that reduced IL-1β expression and limited post-traumatic edema significantly attenuated the manifestation of depressive-like and anxious behavior after TBI. This finding is in line with a report showing P2X7-/- mice exhibited an anti-depressive-like profile and increased responsiveness to antidepressant drugs under basal conditions, as compared to wild-type mice (Basso, et al., 2009). The novel findings presented herein provide support for the notion that acute neuroinflammatory mediators contribute to elevations in ICP as well as influence the development of subsequent neurobehavioral outcomes after TBI.

Several caveats of this study warrant further consideration. Although considered a highly selective P2X7 antagonist, BBG also can inhibit both P2X2 and P2X5, albeit less potently than at P2X7 (Jiang, et al., 2000). Despite our data showing P2X7-/- mice exhibit similar responses to BBG-treated mice, we cannot exclude the possibility that off-target effects on receptors other than P2X7 mediated the beneficial actions of BBG. Similarly, it

remains unclear whether BBG penetrates the blood-brain barrier. We observed a significant accumulation of BBG within the tissue adjacent to the contusion, suggesting BBG could possibly act at the level of the CNS. Nonetheless, we cannot eliminate the possibility that BBG may also act on peripheral immune cells that express P2X7, produce pro-inflammatory mediators, and infiltrate into brain tissue after TBI. Future work by our group using cell-type specific knockout of P2X7 (e.g. astrocyte-specific P2X7 knockout) will attempt to address this issue in detail.

In conclusion, this data suggests a novel, causative role for the low-affinity ATP receptor, P2X7, in the development of cerebral edema and neurological injury after TBI. These findings also identify BBG, a drug that is well-tolerated in humans, in the treatment of cerebral edema and neurological deterioration following TBI using a clinically-feasible therapeutic window. Given the dearth of medical treatment options to limit elevated ICP and reduce co-morbid psychiatric deficits following head trauma, further exploration of P2X7 may be warranted.

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Summary of Dissemination

Type of Dissemination	Citation	Date and Source of Approval for Public Release
Publications	**Kimbler DE**, Murphy M, Dhandapani KM. Concussion and the adolescent athlete J Neurosci Nurs, 43(6), 286-290.	TSNRP PAO 2011
	Kimbler DE, Shields J, Yanasak NE, Dhandapani KM. Activation of P2X7 Promotes Cerebral Edema and Neurologic Injury after Ttraumatic Brain Injury in Mice, Plos One	TSNRP PAO 2012
Poster Presentations	1. **Kimbler DE**, Dhandapani KM. Brilliant blue G (BBG), a P2X7 antagonist, reduced cerebral edema following controlled cortical impact in mice. 2010 National Neurotrauma Society Annual Meeting, Las Vegas, NV	Amedd Center and School PAO 2010
	2. **Kimbler DE**, Shields J, Dhandapani KM. P2X7 inhibition improves neurological outcomes in a murine model of traumatic brain injury. 2011 Society for Neuroscience Annual Meeting, Washington DC.	TSNRP PAO 2011

Reportable Outcomes

Reportable Outcome	Detailed Description
Applied for Patent	None
Issued a Patent	None
Developed a cell line	None
Developed a tissue or serum repository	None
Developed a data registry	None

Recruitment and Retention Aspect	Number
Animals Projected in Grant Application	
Animals Purchased	400
Model Development Animals	0
Research Animals	384
Animals With Complete Data	384
Animals with Incomplete Data	0

*****the descripency in numbers represents mice that died during procedures